MOLECULAR DETECTOR

GOVERNMENT RIGHTS STATEMENT

This invention was developed at least in part using funds and/or facilities of the United States Government, particularly funds provided through grants USDA TH 2002-06107 and CDC E11/CCE021836-01. The United States Government may have certain rights in the invention.

FIELD OF THE DISCLOSURE

The present disclosure concerns an electronic detection device and process for detecting biological agents and biomolecules. More specifically, the present disclosure is directed to an electrical signal generation process, which reports biological interaction events, and provides an electronic device useful for single, multiple and array detection of biological agents and biological molecules including, without limitation, tissues, biopsy samples, cells, yeast, fungus, bacteria, viruses, nucleic acids, proteins, peptides, carbohydrates and other biological molecules.

BACKGROUND

The development of rapid, accurate methods for detecting pathogenic microbes and diagnostic biomolecules are longstanding goals of medical scientific researchers. After September 11th, 2001, detection of biowarfare agents using such methods also has become a high priority for national defense. Various techniques have been developed in an attempt to achieve these goals.

Advanced electronic technologies and devices with their high speed, high density and programmable features show great potential in the development of biosensors. Established semiconductor manufacturing techniques allow large-scale production of reliable devices at low cost, and in multiplexed formats. Moreover, such electronic detection devices can provide near real time data that is valuable for national security and to improve health care and food safety diagnosis.

The link between molecular interaction and electronic signal generation represents an important feature for designing an electronic detection process that can be utilized to selectively detect particular biological target agents. Complicated interactions between different biomolecules, and their poor electrical properties, however, present difficulties for generating electronic signals that can be used to identify biological recognition events and/or provide quantitative data concerning such events. Thus, development of a unique and universal signal generation process and corresponding electrical circuitry are highly desirable for developing electronic biomolecular detectors to meet the challenges of medical diagnostics and national defense.

SUMMARY OF THE DISCLOSURE

The present disclosure is directed to embodiments of a device and a process for generating detectable electronic signals that represent the recognition and interaction of biological agents and biomolecules, whereby, specific biomolecular interaction events are identified with electronics.

Moreover, a universal signal generation process is provided that is designed to interchangeably address the complexity of biomolecular interactions.

In one embodiment, signal molecules that carry an electronic charge and corresponding affinity binding molecules that specifically bind the signal molecules are used to bring an electrical charge to a detection surface. Specifically, signal molecules are captured by affinity binding molecules bound to a detection surface, which results in an increased electronic charge in the vicinity of the surface. The detection device can further include spacer molecules that are bound to the detection surface to form a non-conducting surface that helps alleviate non-specific binding on the detection surface. Electronic circuitry also is provided that is capable of recognizing the presence of the electronic charges induced on the detection surface upon specific binding of the signal molecules by the affinity binding molecules.

Signal molecules, which have a recognition head that specifically binds to an affinity binding molecule on a detection surface and a charged tail that increases the charge at the detection surface, are produced from a signal probe that serves to report the presence of a specific target molecule in a sample, for example, a particular DNA sequence or protein to be detected. The signal probe, which specifically binds to target molecules immobilized in a reaction vessel, includes a recognition component for binding to the target, and a nucleic acid template that codes for the signal molecule. Transcription (and in some embodiments translation) of the template portion of the signal probe (in the presence of appropriate enzymes and substrates) is used to produce the signal molecule. Since the recognition component can be varied to detect different target molecules, while the template portion coding for the signal molecule can be the same regardless of the target, a single type of detection surface having a particular affinity binding molecule that specifically binds the signal molecule can be used to detect a multitude of different target molecules. Furthermore, since the template portion of the signal probe can be used to produce multiple copies of the signal molecule by repeated transcription (and possibly translation), amplification of the signal due to the presence of a target is possible.

In a working embodiment, an *in vitro* transcription DNA template is provided that can be linked to many different biomolecules directly and/or indirectly to form a signal probe. The signal molecules produced by *in vitro* transcription of the DNA template thus can be used for detecting a wide spectrum of biological agents and biomolecules on one or more devices. In a particular embodiment, *in vitro* transcription of a DNA template generates signal molecules that include an RNA aptamer as the recognition head, which signal molecules specifically bind to corresponding affinity molecules on a detection surface. The charged tail (*e.g.*, a poly-A tail) of the signal molecule produced from the template serves to provide a detectable electric charge at the detection surface. Alternatively, peptides having an electronic charge and that bind to affinity molecules on a detector surface can be produced using coupled transcription/translation. Thus, one aspect is to present biological recognition events as changes of electrical properties on a detection surface. Such changes in the electrical properties of a detection surface can be recorded with appropriate electronic circuitry.

The disclosed molecular detector also provides a unique electronic circuitry that can be used to detect the presence of small quantities of electronic charge, such as the induced electronic charge resulting from charged signal molecules binding to affinity molecules at a detection surface. Such circuitry can be used to provide qualitative and quantitative data, and also can be used with other biomolecular detection schemes that produce charge as a component of the detection mechanism.

Current semiconductor fabrication techniques have the capacity to build anywhere from thousands to millions of transistors on a single chip. This capacity facilitates multiple array detection by applying numerous affinity binding molecules on different detection surfaces of a single chip. Consequently, the disclosed method and device for detecting biological agents and molecules on electronic devices can be extended to other field-effect sensing devices, such as silicon or non-silicon nanowires. In a working embodiment, the detection surface includes the gate of a field effect transistor.

Disclosed embodiments of the device can be manufactured using well-established manufacturing processes to produce low cost electronic devices. Since the device can be produced inexpensively relative to many current diagnostic devices, in one embodiment, the device is disposable to avoid cross contamination in the detection process. Moreover, according to yet another embodiment, the device can be integrated with microfluidics to provide a high-throughput detection system that, by virtue of its dimensions, can also enhance the strength of molecular interactions, thereby leading to greater sensitivity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram depicting one embodiment of a disclosed process for detecting a target agent, such as, without limitation, DNA, RNA, proteins, carbohydrates or cells.

FIG. 2 is a schematic diagram depicting the interaction of a signal probe and a capture probe with a target agent.

FIG. 3 is a schematic diagram depicting signal molecule generation.

FIG. 4 is a schematic diagram depicting electrical signal detection of signal molecules.

FIG. 5 is a schematic diagram depicting a flow-through embodiment of the disclosed device in which a reaction vessel and containment area are coupled to permit flow of signal molecules from the reaction vessel to the containment area of a detector surface.

FIG. 6 is a schematic depicting one embodiment of electronics useful for providing a signal path for a charge to propagate from a detector surface, through a region of metal(s) and arrive at the gate of a field effect transistor, the output of which drives amplification electronics and digital analysis circuitry for output on a digital communication channel.

FIG. 7 is a schematic diagram depicting a positive charge comparator circuit.

FIG. 8 is a schematic diagram depicting a negative charge comparator circuit.

FIG. 9 is a schematic diagram depicting a biomolecular charge detector layout.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

The DNA and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 shows the nucleic acid sequence of T7-A30.

SEQ ID NO: 2 shows the 5'-phosphorylated sequence for the T7 Spacer primer.

SEQ ID NO: 3 shows the nucleic acid sequence for T7-R primer.

SEQ ID NO: 4 shows the DNA sequence for the T7-rPS2.M Template.

SEQ ID NO: 5 shows the complementary DNA sequence for the T7-rPS2.M Template.

SEQ ID NO: 6 shows the DNA sequence for the T7-rPS2.M-Poly A Template.

SEQ ID NO: 7 shows the complementary DNA sequence for the T7-rPS2.M-Poly-A Template.

SEQ ID NO: 8 shows the RNA sequence for rPS2.M.

SEQ ID NO: 9 shows the RNA sequence for rPS2.M-Poly A.

SEQ ID NO: 10 shows the nucleic acid sequence for the T7-P22B Top Sequence.

SEQ ID NO: 11 shows the nucleic acid sequence for the T7-P22B Bottom Sequence

SEQ ID NO: 12 shows the nucleic acid sequence for the amine modified first Spacer-P22B primer. The sequence is amine modified at the 5' phosphate with an amine modifier with a 6 carbon spacer.

SEQ ID NO: 13 shows the nucleic acid sequence for the thio-modified first Spacer-P22B primer. The sequence is thio-modified at the 5' phosphate with a thio modifier with a 6 carbon spacer.

SEQ ID NO: 14 shows the nucleic acid sequence for the second P22B-R primer.

SEQ ID NO: 15 shows the nucleic acid sequence for the T7 Promoter.

SEQ ID NO: 16 shows the nucleic acid sequence for a 5' Stem Loop and ribosome binding site sequence.

SEQ ID NOs: 17-23 show the amino acid sequences of the following epitope tags: HIS, c-MYC, HA (haemagglutanin), VSV-G, HSV, V5, and FLAG.

SEQ ID NO: 24 shows the nucleic acid sequence for a 3' Stem Loop.

SEQ ID NO: 25 shows the nucleic acid sequence for a T7 Terminator.

SEQ ID NO: 26 shows the amino acid sequence for a water soluble 10-mer peptide used in certain embodiments.

SEQ ID NO: 27 shows the nucleic acid sequence for a 30 base pair recognition component designed to hybridize to pBluescript SK +. The 5' end is phosphorylated.

SEQ ID NO: 28 shows the nucleic acid sequence for the capture probe. The 5' end is biotinylated.

DETAILED DESCRIPTION

Embodiments of the biosensor disclosed herein comprise a unique universal signal generation process structure in combination with a unique electronic charge detector to detect various biological agents and biomolecular targets of interest. The signal generation process is able to detect any biological agent or biomolecule. The electronic charge detector detects the presence of small quantities of electronic charge, such as a charge as low as about 150 electron equivalents. These features and others are described below.

I. General Discussion

In the following discussion and the examples that follow, all abbreviations and terms have the meanings ascribed to such abbreviations and terms by those of ordinary skill in the art, unless otherwise noted. In case of conflict, the meanings provided herein control.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. Thus, the materials, methods, and examples provided herein are illustrative only and not intended to be limiting.

The singular terms "a," "an," and "the" include plural referents unless the context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "comprises" means "includes." Hence, "comprising A or B" means including A or B, or A and B, unless the context clearly indicates otherwise.

A. Overview

The disclosed method and device incorporate components that function as a system to detect particular biomolecules and other agents of interest with great sensitivity. Biomolecules and agents of interest that can be detected using the disclosed method and device include, without limitation, peptides, polypeptides, proteins, sugars, polysaccharides, carbohydrates, glycoproteins, lipids, lipoproteins, antigens, antibodies, receptors, ligands, enzymes, nucleic acids, oligonucleotides, DNA, RNA, cells, biopsy samples, tissues, yeast, fungus, bacteria, viruses, etc., and are collectively referred to herein as "biomolecules."

In one embodiment, a device for detecting biomolecules is provided that includes a detection surface electronically coupled to an electronic circuit, a molecular layer immobilized on the detection surface and a signal molecule in a containment area. The molecular layer immobilized ("immobilized" means attached directly or indirectly, covalently or non-covalently, or transiently or permanently) on the detection surface includes a mixture of an affinity binding molecule and a spacer molecule. The molecular layer can cover part of the detection surface or substantially the entire detection surface, and can be a monolayer or include multiple layers of molecules. The containment area can be any type of barrier, space, path or container (such as a well, hydrophilic or hydrophobic patch, or channel) that maintains contact between the signal molecule (such as a signal molecule

dissolved or suspended in a liquid sample) and the detection surface. Such contact need not be continuous, just of sufficient time for the signal molecule to bind to the affinity binding molecule immobilized on the detection surface.

The disclosed signal molecule includes a recognition head and an electrically charged tail, wherein the recognition head of the signal molecule specifically binds to the affinity binding molecule on the detection surface. The terms "specific binding," "specifically binding" and "specifically bound" collectively refer to the situation where two or more molecules or molecular complexes bind to one another to the substantial exclusion of binding with other components that may be present. Although binding constants can be used in the selection of the affinity binding materials (for example, a binding constant of greater than about 10^3 L/mol, such as a binding constant of greater than about 10^6 or 10^9 L/mol), the primary selection is based on the ability of the binding between the two components to generate a statistically significant signal above background using the disclosed device, which is not detected in the absence of one or more of the interacting molecules. Examples of specific binding include antibody/antigens interactions, streptavidin/biotin interactions, RNA aptamer/binding partner interactions (such as between an aptamer and a protein such as hemin), lectin/sugar interactions, nucleic acid hybridization, and the like

The electronic circuit of the device is configured to determine the presence of the signal molecule specifically bound by the affinity binding molecule that is immobilized on the detection surface.

Typically, the signal molecule is produced from a signal probe. Signal probes include a recognition component and a signal template component. The recognition component specifically binds, either directly or indirectly, a target to be detected, and the signal template component codes for the signal molecule. As used herein, "codes for" means that the template can be used to direct the synthesis of a particular signal molecule under the appropriate reaction conditions. For example, a DNA template codes for a particular RNA sequence that can be synthesized by a transcription reaction (using an appropriate RNA polymerase and NTPs), wherein the transcription reaction is directed by the DNA template. Furthermore, an RNA template (or an RNA produced from a DNA template) can be translated into a polypeptide sequence. Thus, an RNA or DNA sequence can also "code for" a particular polypeptide sequence.

In some embodiments, the recognition component of the signal probe includes a biomolecule, and the signal template component comprises a DNA template. Examples of biomolecules that can form the recognition component of the signal probe include, without limitation, antibodies, antigens, enzymes interactive with particular substrates, nucleic acids, proteins, polypeptides, substrates that specifically bind particular polypeptides, receptors that specifically bind particular ligands, and ligands that specifically bind particular receptors.

In particular embodiments, signal molecules are produced through *in vitro* transcription of a DNA template linked to the biomolecule. In others, signal molecules are produced by transcription (for example, *in vitro* transcription) of a DNA template to produce an RNA transcript, which is then translated (for example, by *in vitro* translation) into a polypeptide sequence.

In some embodiments, the detection surface of the disclosed device includes a conductor, for example, a metal such as gold, platinum, copper, aluminum, tin, or silver. In other embodiments, the detection surface includes a semiconductor material such as silicon, silicon dioxide or polysilicon.

The affinity binding molecule of the device can be directly or indirectly immobilized on the detection surface, and the immobilization can be covalent or non-covalent. Affinity binding molecules that specifically bind to the target (directly or indirectly) include, for example, organic molecules such as mercaptoundecane, mercaptoundecanoic acid, or mercaptoundecylamine and biomolecules such as RNA aptamers, proteins (such as antibodies and lectins), metalloporphyrins such as hemin, polyelectrolytes (such as chitosan, polylysine, alginate, or a polyphosphate), nucleic acids (such as thymine, uracil, poly-T, poly-U, poly-G, poly-A, poly-C or other polynucleic acids). In certain particular embodiments, the affinity binding molecule interacts with the signal molecule to provide enhanced enzymatic activity, for example, if the affinity binding molecule is hemin, the signal molecule can interact with the hemin to enhance enzymatic activity and amplify the signal via the enhanced enzymatic activity.

The spacer molecules can also be directly or indirectly, covalently or non-covalently, or transiently or permanently immobilized on the detection surface of the disclosed device, and can be, for example, a small organic molecule or an organic polymer. In some embodiments (particularly where the detector surface comprises gold), the small organic molecule can be a mercaptoalcohol such as mercaptohexanol. In particular embodiments, the organic polymer includes polyethylene glycol.

As mentioned above, the signal molecule coded for by the signal probe includes a recognition head that binds to the affinity binding molecule on the detection surface. In general, the same types of biomolecules and organic molecules that can be used for the affinity binding molecule can be used for the recognition head of the signal molecule. In particular embodiments, the signal molecule includes an RNA transcript (such as an aptamer), a tag peptide (such as a poly-histidine peptide, a FLAG peptide, a HA (hemagglutinin) peptide, or a myc peptide), a biotinylated antigen, a polynucleic acid or a polypeptide.

The other portion of the signal molecule is the electrically charged tail, which can be any electrically charged sequence (positive or negative), for example, a polynucleotide or polypeptide sequence, or some combination thereof. In working embodiments, the electrically charged tail comprises a poly-A tail, which is conveniently produced using poly-A polymerase, an enzyme that continues to lengthen the tail without the need for a long DNA sequence coding for the same. Also in a working embodiment, a signal probe that codes for a signal molecule including an RNA aptamer recognition head and an electrically charged poly-A tail is provided. By selection of different aptamers (or by selection of a first molecule that specifically binds to a target and an aptamer that binds to the first molecule), different targets can be detected.

In particular embodiments, the disclosed device includes a detection surface that is electronically coupled directly or indirectly to a gate of a transistor (such as an n-MOS FET or a p-MOS FET), where the transistor drives electronics that produce qualitative or quantitative data

reflective of binding of the signal molecule to the affinity binding molecule immobilized on the detection surface. In other particular, embodiments the electronic circuit includes a plurality of n-MOS and p-MOS devices. In more particular embodiments, the electronic circuit further includes a conductor path with a first end electronically coupled to the detector surface, a polysilicon gate of a field effect-transistor (FET) electronically coupled to a second end of the conductor path, amplifier electronics electrically coupled to the FET, and digital analysis circuitry electrically coupled to the amplifier electronics. Typically, the digital analysis circuitry is configured to perform at least accounting, ratio, summation, and threshold operations, and combinations thereof. Furthermore, in embodiments including a polysilicon gate, the initial bias charge of the polysilicon gate is held above a threshold charge for a MOSFET transistor such as an n-MOS FET or a p-MOS FET. In other particular embodiments, the transistor of the electronic circuit can include an ultra low power transistor with low threshold voltage (positive or negative) near to or even zero (for example, a voltage of from 0 V to 0.001 V, such as a voltage of from 0 V to 10^{-6}V or from 0 to 10^{-9}V. A reference voltage can also be provided to the circuit.

Also provided are devices that couple the containment area of the device to a reaction vessel in which specific binding reactions and reactions used to generate signal molecules are conducted. Alternatively, it is possible for the containment area to also serve as a reaction vessel. In more particular embodiments, connections between the containment area and the reaction vessel can be made using microchannels. Such embodiments employ the principles and techniques of the field of microfluidics. Microfluidics-based devices typically make use of ten to several-hundred micrometer wide channels, and micropumps, electroosmotic flow, integrated valves and mixing devices control liquid movement through the channel network. In the disclosed device, such components can be used to deliver the signal molecule to the containment area of the device. Such components may be prepared in a substrate (such as silicon or plastic) using well-known micromachining and microfabrication methods. The terms "micromachining" and "microfabrication" both refer to any number of techniques which are useful in the generation of microstructures (structures with feature sizes of sub-millimeter scale). Such technologies include, but are not limited to, laser ablation, electrodeposition, physical and chemical vapor deposition, photolithography, and wet chemical and dry etching. Related technologies such as injection molding and LIGA (x-ray lithography, electrodeposition, and molding) are also included. Most of these techniques were originally developed for use in semiconductors, microelectronics, and Micro-ElectroMechanical Systems (MEMS) but are applicable to the presently disclosed device as well. Fluid channels in such microscale devices can serve as reaction vessels (or interaction vessels) that can house respectively different interactions such as reactions or binding events. In addition, each wall of a fluid channel may form a reaction vessel. In a typical assembly, each individual reaction vessel can house a different event (e.g., a different reaction or binding event). In other embodiments, the different reaction vessels may house the same types of events.

Also disclosed is a method for detecting a target in a sample. The target can be any organic molecule, inorganic substance or biomolecule as defined above. The method includes immobilizing

the target in a reaction vessel. This can be accomplished by non-specific binding of the target to the reaction vessel, or through the use of a capture probe. A capture probe includes an attachment component that can bind (covalently or non-covalently, specifically or non-specifically) to the vessel and a capture component that specifically binds (directly or indirectly) to the target and serves to link the target to a surface of the reaction vessel. The target is contacted with a signal probe.

The signal probe specifically binds (directly or indirectly) to the target through its recognition component, thereby also binding the signal template component of the signal probe to the target. The signal template component is then used to generate the signal molecule (and, in some embodiments, more than one signal molecule or copies of a signal molecule are produced from a single template), which has a recognition head and an electrically charged tail. The signal molecule is detected at a detection surface, where the recognition head of the signal molecule specifically binds to an affinity binding molecule immobilized on the detection surface. The charged tail of the signal molecule serves to increase the charge at the detection surface, thereby making it easier to detect binding of the signal molecule, which indicates the presence of the target in the sample. In some embodiments, the method further includes removing any signal probe that is not specifically bound to the target immobilized in the reaction vessel.

FIG. 1 illustrates one embodiment of the disclosed method and device that includes the use of both capture probes and signal probes. One embodiment of a capture probe includes an attachment end for coupling the probe to a reaction vessel, and a target capture component designed to capture the target and immobilize it in the reaction vessel. The signal probe, which recognizes and signals the presence of specific biological materials, is used to generate signal molecule(s) that are reflective of the presence of specific biological materials. The signal molecules also may amplify the signal so that the charge can be detected, such as at the gate of a semiconductor device.

Target capture, and signal generation and amplification, can occur outside of the semiconductor detector in a reaction vessel, such as in a test tube, a well (for example, a well in a microtiter plate or etched into a substrate, such as a silicon substrate), a chamber (such as a chamber formed in a plastic or other substrate) or any other type of reaction vessel. Then, signal molecules are introduced into a containment area around the detector for specific detection. Transfer of the signal molecules from the reaction vessel to the containment area of the detector can be automated, for example, through the application of fluidics, such as microfluidics (*see*, for example, FIG. 5). Alternatively, the specific binding interactions and signal generation/amplification reactions can both occur in a containment area around the detector, where introduction and removal of solutions can be automated, for example, through application of microfluidics.

Signal molecules in the containment area come in contact with the detector surface, where they are specifically captured by an affinity binding molecule, thereby generating a charge at the detection surface that can be detected. The signal molecules typically will have a recognition head, which specifically binds with the affinity binding material, and a charged tail, which serves to increase and amplify the electric signal through intrinsic charge and/or enzymatic activity, for example, enzymatic reduction of hydrogen peroxide (such as catalyzed by hemin).

In one embodiment, the signal probe is a DNA template that is used to generate through *in vitro* transcription a specific RNA aptamer that serves as the signal molecule. An aptamer is defined herein as an oligonucleotide sequence such as a double stranded or single stranded DNA or RNA molecule that binds to specific molecular targets, such as a protein or metabolite. An RNA aptamer can include a recognition head which specifically reacts with the affinity binding materials and a charged polynucleotide which serves as the charged tail. The signal molecule is captured on the surface of the detector using a specifically designed affinity binding biomolecule.

In a second embodiment of the signal probe, the DNA template is designed to produce a specific tag peptide having a recognition head and a charged tail. In specific examples of such probes, the DNA template includes codons for a specific (and, in some instances, commercially available) affinity peptide tag or combination thereof, such as His-tag, myc-tag, Flag-tag, or HA-tag, which serves as the recognition head of the signal molecule. Optionally, the sequence encoding the tag can be repeated, so that the translated peptide contains multiple copies of the tag. In some embodiments, peptide spacer(s) are included between each of the two or more tags. In addition, codons for an amino acid with a charged side chain at a particular pH are included to provide a charged tail, which provides positive or negative charge to the peptide. The charged tail can include both charged and uncharged amino acids in various combinations, so long as there is an overall (net) charge to the tail.

In a third embodiment of the signal probe, biotin is used to specifically label a mixture of target antigens in the test sample. Subsequently the biotinylated antigens are captured on the semiconductor gold gate through the use of specific antibodies for the identified target antigens. Streptavidin is then added which specifically binds to the bound biotin labeled antigens on the gold gate. To amplify the signal and bring a charge to the detection surface, biotinylated DNA (negative charge) or biotinylated polylysine (positive charge) can be added, which will bind to the streptavidin/biotin labeled antigens on the gate surface. This yields an appropriate positive or negative charge that can be detected on, for example, a transistor surface.

Affinity binding peptides/proteins and nucleosides have been designed for use on a semiconductor gold gate. The purpose of these materials is to bring amplified, charged signal molecules sufficiently proximate to the sensor gate so that the charge can be detected. Embodiments of the affinity binding biomolecule include a peptide, antibodies, a 5'-thio-oligonucleotide, and combinations thereof.

B. Preparation of Signal Probe

Again with reference to FIG. 1, in a first disclosed embodiment, a signal probe generally consists of two components: a DNA signal amplification component (DNA-T); and a target recognition (RC) component. The DNA template (DNA-T) used for the initial signal probe amplification component was designed to contain 80 base pairs and consists of four components: an EcoRI restriction site at the 5' end and a BamHI restriction site at the 3' end (designed to provide "sticky ends"); a T7 promoter region; and an RNA transcript region. Three oligomers were designed,

and these oligomers were chemically synthesized by a commercial vendor (Integrated DNA Technologies, Inc., Coralville, IA) and included the following:

T7-A30 Sequence (77 Bases)

(SEQ ID NO: 1)
5'-CCG AAT TCT AGG GTA ATA CGA CTC ACT ATA GGG AAC AAA AAA AAA AAA AAA AAA AAA AAA AAA AAT GCA GGA AAG AG -3'

Spacer-T7 (20 bases) (Primer 1)

(SEQ ID NO: 2)
5'- /Phos/CCG AAT TCT AGG GTA ATA CG -3'

T 7-R (18 bases) (Primer 2)

(SEQ ID NO: 3)
5'- CTC TTG GAT CCT GCA TTT -3'

The above T7-A30 oligomer was amplified using PCR with the listed primers for 30 cycles. The resulting 77-base pair DNA template was purified using a standard PCR purification kit (Qiagen, Inc., Valencia, CA).

The resulting DNA Template was tested for RNA transcription using a standard *in vitro* T7 RNA polymerase protocol with reaction times of up to 45 minutes (*see*, for example, Sambrook, J. and D.W. Russell, "Synthesis of single-stranded RNA probes by in vitro transcription", Molecular Cloning, 3rd edition, Vol. 2, Protocol 6, 2001, pp. 9.29-9.37). Gel electrophoresis showed the generation of the 45-base RNA aptamer. RNA transcription also was carried out with the addition of both the T7 RNA polymerase and Poly A polymerase. This resulted in the formation of a RNA aptamer with a poly A tail, again confirmed using gel electrophoresis.

The above-described T7-A 30 DNA template is designed to produce a signal molecule consisting of an RNA aptamer that includes a recognition head with a charged tail comprising polyadenosine units. This signal molecule can be detected on a detector surface using uridine, thymidine, oligodeoxythymidine (such as oligo dT with about 20-30 bases) or combinations thereof, as the affinity binding material bound to the detector surface. Uridine and thymidine can be attached to the gold surface of the gate by a thiol linkage, as described below. The signal probe that is used to generate the signal molecules can be modified by changing its DNA sequence to produce a change in the signal component of the probe and thus a change in the signal molecule itself.

Using this approach, a second probe was synthesized to produce the RNA aptamer, rPS2.M, an 18 nucleotide oligomer with a strong binding affinity for hemin. *See*, Travascio *et al.*, *Chemistry and Biology* 5:505-517, 1998; *Chemistry and Biology* 6:779-787, 1999; and *J. Amer. Chem. Soc.* 123:1337-1348, 2001, which are all incorporated by reference herein. Two DNA templates of the signal molecule were produced. The T7-rPS2.M DNA Template contains the T7 promoter sequence and the PS2.M sequence, while the T7-rPS2.M-poly A DNA Template contains an additional section to produce poly A. The templates were designed and chemically synthesized by a commercial vendor (Integrated DNA Technologies, Inc., Coralville, IA) with sequences as follows:

T7-rPS2.M DNA Template

(SEQ ID NO: 4)
5'-GGT AAT ACG ACT CAC TAT AGG GAA GTG GGT AGG GCG GGT TGG -3'
(SEQ ID NO: 5)
3'-CCA TTA TGC TGA GTG ATA TCC CTT CAC CCA TCC CGC CCA ACC-5'

T7-rPS2.M-Poly A DNA Template

(SEQ ID NO: 6)
5'-GGT AAT ACG ACT CAC TAT AGG GAA GTG GGT AGG GCG GGT TGG AAA AAA AAA AAA -3'
(SEQ ID NO: 7)
3'-CCA TTA TGC TGA GTG ATA TCC CTT CAC CCA TCC CGC CCA ACC TTT TTT TTT TTT-5'

rPS2.M (18 bases)

(SEQ ID NO: 8)
5'- GUG GGU AGG GCG GGU UGG -3'

rPS2.M-Poly A (30 bases)

(SEQ ID NO: 9)
5'- GUG GGU AGG GCG GGU UGG AAA AAA AAA AAA -3'

The DNA Templates were tested for RNA transcription using a standard *in vitro* T7 RNA polymerase protocol with reaction times of up to 45 minutes (see, for example, Sambrook, J. and D.W. Russell, "Synthesis of single-stranded RNA probes by in vitro transcription", Molecular Cloning, 3rd edition, Vol. 2, Protocol 6, 2001, pp. 9.29-9.37.). Gel electrophoresis showed the generation of the 18-base RNA aptamer from the T7-rPS2.M DNA Template while the T7-rPS2.M-Poly A DNA Template produces an RNA aptamer that includes a recognition head with a charged tail comprising polyadenosine units. As with the first signal molecule, this signal molecule can be bound to the detector surface using uridine, thymidine, oligodeoxythymidine (oligo dT with 20-30 bases) or combinations thereof, as the affinity binding materials. Hemin modified gold surfaces can also be used for the affinity binding surface for both rPS2.M and rPS2.m-poly A as described below.

A third signal probe was designed for the production of RNA aptamers that attach to a peptide that serves as the affinity binding materials. An initial example is a P22N21 peptide, a 212 amino acid peptide that is an arginine-rich peptide (*see*, Austin *et al.*, *J. Am. Chem. Soc.* 124:10966, 2002, which is incorporated by reference herein). The P22N21 peptide has a high affinity for a specific P22 Box RNA aptamer. The DNA template for this probe was designed and cloned in the pUC19 cloning vector. The DNA template used for the P22 signal probe contains ~80 base pairs and consists of 4 components: an EcoRI restriction site at the 5' end; a BamHI restriction site at the 3' end (designed to provide "sticky ends"); a T7 promoter region; and an RNA transcript region coding for the P22 Box aptamer. Oligomers were designed, and these oligomers were chemically synthesized by a commercial vendor (Integrated DNA Technologies, Inc., Coralville, IA). Their sequences follow.

T7-P22B Top Sequence (47 Bases)

(SEQ ID NO: 10)
5'-AAT TCG GTA ATA CGA CTC ACT ATA GGG AAG GCG CTG ACA AAG CGC CG -3'

T7-P22B Bottom Sequence (47 Bases)

(SEQ ID NO: 11)
5'-GAT CCG GCG CTT TGT CAG CGC CTT CCC TAT AGT GAG TCG TAT TAC CG -3'

Spacer-P22B-am-F (20 bases) (Primer 1) (Amine modified)

(SEQ ID NO: 12)
5'- /5AmMC6/AAC GAC GGC CAG TGA ATT CG -3'

Spacer-P22B-thio (20 bases) (Primer 1) (Thio modified)

(SEQ ID NO: 13)
5'- /thioC6/AAC GAC GGC CAG TGA ATT CG -3'

P22B-R (20 bases) (Primer 2)

(SEQ ID NO: 14)
5'- ACT CTA GAG GAT CCG GCG CT -3'

The above T7-P22B top and bottom strands were annealed to form a double strand DNA fragment. Primer 1 was modified at the 5' phosphate with either an amine or a thio with a 6 carbon spacer thus allowing the conjugation of this signal molecule to the recognition component of the signal probe. This fragment was used as a template for testing RNA transcription, which was done using a standard *in vitro* T7 RNA polymerase protocol with reaction times of up to 45 minutes. Gel electrophoresis showed the generation of the RNA aptamer. AmpliScribe™ T7-Flash™ Transcription kit (EPICENTRE, Madison, WI) also was used for amplification and significantly reduced the time needed for amplification. The T7-P22 DNA fragment cloned into the pUC19 vector can be used as a template to generate signal probes with varying spacer lengths by using forward primers in different locations. The resulting signal molecule includes the P22 Box RNA aptamer as the recognition head and poly A as the charged tail.

A fourth signal probe is designed for the production of tag peptides. The DNA template (DNA-T) used for this embodiment is designed to contain 5 components in addition to SpeI restriction sites at the 5' and 3' ends. It includes a T7 promoter region; a 5' stem loop and rbs sequence; a tag peptide and charged polypeptide sequence; a 3' stem loop sequence; and a T7 terminator sequence. Five oligomers are designed and are chemically synthesized. The overall construct and sequences for a representative DNA-T are shown below:

Overall DNA Template (DNA-T) Construct

T7 promoter-----5'stem loop and rbs-----Peptide tag and polypeptide ORF------3' stem loop -----T7 terminator (can be modified to include SpeI restriction sites)

Sequences

T7 Promoter

(SEQ ID NO: 15)
TAATACGACTCACTATAGGGAGA

5' Stem Loop and *rbs* Sequence

(SEQ ID NO: 16)
CCACAACGGTTTCCCAATAATTTTGTCTAGATAGAGCCCTCAA***AAGGAG***TTTGACAT

General Formula/Structure of Sequence Encoding the Peptide Tag and Polypeptide ORF

(start)(peptide tag)$_m$(charged amino acid tail)$_n$(stop)

The "start" can be, for instance, ATG. The term "peptide tag" refers to the nucleic acid sequence encoding the particular tag peptide (for instance, HA-tag, His-tag, Flag-tag, myc-tag, etc.). With reference to this general formula, "m" indicates the number of amino acid codons encoding the peptide tag present, and typically varies from about s to about 6 (*e.g.*, in a typical His-tag) to 8 (*e.g.*, a typical Flag-tag), and so forth, depending on the length of the tag used, and, in some embodiments, the number of repetitions of the tag included in the construct. The following table provides sequences for representative, non-limiting example epitope tags for which specific corresponding antibodies are commercially available:

Tag	Sequence	SEQ ID NO:
HIS	HHHHHH	17
c-MYC	EQKLISEEDL	18
HA	YPYDVPDYA	19
VSV-G	YTDIEMNRLGK	20
HSV	QPELAPEDPED	21
V5	GKPIPNPLLGLDST	22
FLAG	DYKDDDDKG	23

The phrase "charged amino acid tail" in the general formula refers to the codon(s) for the amino acids in the charged polypeptide tail. In reference to that, "n" indicates the number of amino acids in the tail, and typically varies from about 5 to about 50, and more typically is from about 5 to about 20. More specifically, the overall (net) charge of the tail is contemplated to be a net charge of about 5-25, and in some instances will be about 20. The amino acid(s) selected for the tail (including optionally both charged and uncharged amino acids), and numbers thereof, can be varied to produce the amount and type of charge desired. Amino acids with different charge are well known to those of ordinary skill in the art. The "stop" is any stop codon, for instance, TAG, TAA, or TGA.

Thus, in one specific example, a nucleotide sequence encoding a peptide tag and polypeptide ORF would contain a start codon, a sequence encoding the HIS tag, a series of codons for lysine (*e.g.*, AAG), for instance 10-25 lysines, to form the tail, and a stop codon. Other sequences could be generated using the guidelines and teachings provided herein.

3' Stem loop

(SEQ ID NO: 24)
CCGCACACCAGTAAGGTGTGCGG

T7 terminator

(SEQ ID NO: 25)
CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTA

The resulting DNA Template is tested for RNA transcription and peptide translation using a standard coupled *in vitro* transcription/ translation protocol using *E. coli* S30 cellular extracts. This results in the formation of a tag peptide with a polypeptide tail where the tag peptide serves as the recognition head and the polypeptide serves as the charged tail. The above-described template is designed to produce a positively or negatively charged signal molecule that can be detected on the detector surface using anti-tag antibodies, or nickel or other affinity binding materials. Antibodies can be attached to a gold surface of a gate by a thiol linkage as described below.

As described above, the signal probe can be modified by changing the DNA sequence to produce a change in the signal component or through a change in the recognition component to allow the specific recognition of a variety of biological materials (cell, DNA, protein, etc.). The advantage of this embodiment is that the sequences of the peptides are well-known and the anti-tag antibodies can be purchased from a commercial vendor.

The recognition component of the signal probe can include proteins, peptides, or DNA/RNA aptamers, which have been designed to interact specifically with the target. These compounds can be directly conjugated to the DNA template to complete the signal probe. In an initial embodiment, the signal component is linked to the recognition component using commercial linkers that allow the direct covalent linking of the DNA of the signal component to protein and/or RNA in the recognition component. Antibodies are useful recognition components due to their commercial availability and known interactions. To assist in the linkage, the DNA template can be modified to include an amine or a sulfhydryl at the 5' end. A more robust linker currently is being designed using Versalinx™ technology, which is based on the interaction of phenylboronic acid (PBA) and salicylhydroxamic acid (SHA) (*see*, Stolowitz *et al.*, *Bioconjugate Chem.*, 12:229-239, 2001). SHA- and PBA-modifying reagents are synthesized for conjugating these compounds to peptides/proteins and the DNA template to allow linkage through the SHA:PBA interaction. This includes the addition of linker carbon extensions to prevent steric interference with the other components of the capture/signal probes.

In a third embodiment of the signal probe, a signal probe was designed using biotin to specifically label a mixture of target antigens in the test sample. The concept was verified using a commercially available biotin labeling kit (N-hydroxysuccinimide-Polyethylene Oxide-Biotin, NHS-PEO4-Biotin, Pierce Biotechnology, Rockford, Ill.) to label the outer membrane proteins of E. coli. The biotin labeled antigens can be directly captured on the detector surface by the use of specific antibodies as the affinity binding materials as described below. The advantage of this embodiment is that it does not require the amplification of the signal molecules and can be applied to a number of different antigens.

C. Preparation of Capture Probes

The capture probe is designed to interact with and capture the target and subsequently facilitate the interaction of the signal probe with the target. In most cases the target molecule will be immobilized in a reaction chamber, such as a test tube, a column or a plate well. As a result, the capture probe is advantageously linked to such a chamber, for example, by being attached to at least a portion, and perhaps substantially completely coating, chamber surfaces, such as glass slides, columns, or magnetic beads. Examples of this capture probe interaction include, without limitation, the Streptavidin:Biotin interaction or the PBA:SHA interaction. Either component of the pair can be attached to the stationary phase (such as a surface of a reaction vessel), and the other interactive reactant attached to the capture probe.

As with the recognition component of the signal probe, the capture probe can be designed to attach to the target at a specific 3-dimensional site, in most cases, at a different interactive site than that of the signal probe. As with the recognition component, the biomolecules used for the capture probes will, in most cases, be proteins, peptides or DNA/RNA aptamers. In some cases, the capture probe serves to immobilize the target to allow interaction with the signal probe without the need for a specific interaction. In these situations, the interaction may be a nonspecific binding as can be achieved through a test well, membranes and other materials. It also is possible that the sample could be a biopsy or a slice of animal (*e.g.*, mammalian) or plant tissue or cell(s) affixed to a glass slide using customary techniques.

D. Affinity Binding Biomolecules and Spacer Molecules

A molecular monolayer coupled to a detection surface includes spacer molecules and affinity binding biomolecules. The purpose of the affinity binding materials is to bring the amplified, charged signal molecules proximate to the sensor gate so that the charge can be detected. On the other hand, the spacer molecules are substantially non-conductive molecules, and are used to prevent non-specific binding on the detection surface. These materials form a layer, such as a self assembled monolayer, on the surface of the gold gate. In most cases the spacer molecules are small, uncharged organic molecules or organic polymers, such as polyethylene glycol or mercaptohexanol. The small molecular size and lack of charge of these spacer molecules prevents competition with the larger and possibly charged affinity binding molecules. In certain embodiments, the spacer molecules are covalently bonded to the detection surface. For example, mercapto-alcohols can be covalently linked to a gold surface through the sulfhydryl group. In one embodiment, mercaptohexanol is effectively linked to a gold sensor gate and prevents charge transfer from ionic solutions to the sensor gate surface.

Affinity binding molecules recognize and capture the signal molecules and can be formed of materials such as biomolecules, metals and/or organic molecules that specifically bind the signal molecules. Some embodiments include affinity binding peptides, proteins and nucleosides for use on a semiconductor gate electrode. The affinity binding materials specifically bind the recognition head of the signal molecules. As with the spacer molecules, the affinity binding materials can be bound to

the gate surface using conventional bioconjugation techniques (*see*, for example, Hermanson, *Bioconjugate Chemistry*, Academic Press, 1996 and Lobert *et al.*, *Sensors and Actuators B*, 92:90-97, 2003). In the case of gold as the gate surface, biomolecules can be attached to the surface through sulfhydryl groups attached to the biomolecules. Thus, with proteins or peptides, a cysteine can be included in the sequence to allow the reaction of its free sulfhydryl with the gold surface. In the case of DNA or RNA, these materials can be thiolated and attached via the free sulfhydryl [see, Hermanson, G.T., *Bioconjugate Chemistry*, Academic Press (1996)]. The biomolecules can also be conjugated to other sufaces such as aluminum oxide or silicon oxide through amine, aldehyde, thiol, siloxane or other functionalization of the oxide surfaces. The biomolecules can also be functionalized with amine, aldehyde, thiol, or other such groups to allow conjugation using commercially available linkers or by direct conjugation to the surface. For example, thiolated DNA, RNA or proteins as described above can be linked to an amine group on the surface through the use of a cross-linker with a maleimide and succinimide ester moiety such as Sulfo-SMCC (Peirce Biotechnology, Rockford, IL) while aminated RNA, DNA or proteins can be directly linked to a gate surface modified with aldehyde groups. Affinity binding molecules are selected based generally on their affinity for a given signal molecule. For the T7-A30 probe and the rPS2.M-Poly A probe described above, 5'-thiol-oligo dT and/or 5'-thiol oligo dU can be used as the affinity binding materials. For the T7-P22B probe, a specific P22 peptide, a 22 amino acid peptide sequence with high affinity for the specific RNA aptamer (P22B RNA) can be used. The 22-amino acid is an arginine-rich peptide that has been modified by the addition of glycine and cysteine to allow conjugation to the gold surface.

In addition, a 10-amino acid peptide was designed and synthesized. The 10-amino acid peptide is water soluble and allows closer contact of the charged aptamer to the semiconductor surface. The sequence is CGGGSRYNHD (SEQ ID NO: 26). The cysteine residue allows bonding to a gold surface. SELEX (Systematic Evolution of Ligands by Exponential Enrichment) is used to optimize the RNA aptamer sequence for binding to this peptide and the needed DNA sequence is incorporated into the signal probe.

For a tag peptide the affinity binding biomolecule can be an anti-tag antibody, nickel or other affinity binding materials attached to the gold surface. For the biotin-antigen signal molecule, antibodies specific to the target antigen are selected and bound to the gold gates through direct adsorption, an activated surface process or using bifunctional linkers.

Hemin can be used as the affinity binding material for both the rPS2.M-Poly A probe and the rPS2.M probe. Hemin is attached to the gate surface through the use of direct linkage to mercaptoundecylamine (MUAM). MUAM is first used to form a self-assembled monolayer using mercaptoethanol as a spacer molecule on the gold gate surface and is then reacted with hemin which has been activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The reaction results in covalently linking the hemin to the MUAM monolayer. Experiments showed that the PS2.M DNA oligomer with and without a poly A tail and the rPS2.M RNA with and without a poly A tail can bind to the bound hemin on the gate surface.

In some cases the affinity binding material can consist of polyelectrolytes such as polylysine, polyphosphates, alginates and chitosan that can be bound to the gate and serve as non-specific binding materials for oppositely charged materials. Chitosan can also serve the purpose of providing a sol-gel framework that allows the detection to occur in a sol-gel state rather than in solution.

The interaction between the affinity binding molecules and the signal molecules results in a change in the electrical charge of the gate. In the case of the RNA aptamers with the attached polyA tails having negative charges, the interaction results in increased negative charge on the detection surface or surfaces while with the tag peptides, there can be an increased positive or negative charge due to the positively or negatively charged polypeptide tails of the signal molecules. In the case of the biotin labeled signal molecules, biotinylated antigens can first be captured at the gate surface by specific antibodies bound to the surface. Streptavidin is then added to react with the biotin, and, subsequently, biotinylated DNA (negative charge) or biotinylated polylysine (positive charge) is added to bind to the streptavidin:biotin labeled antigens on the gate surface, thus bringing the appropriate positive or negative charge to the detector surface, such as a transistor surface.

Charge achieved from the rPS.M signal molecules can be further amplified on the gate surface through the addition of hemin that binds to the signal molecule and adds charge. This signal can be further amplified by taking advantage of the peroxidase activity of the rPS.M aptamer sequence. Experiments have shown that PS2.M DNA with and without a poly A tail as well as the rPS2.M RNA with and without a poly A tail have significant peroxidase activity in the presence of hemin. Through the addition of hydrogen peroxide and a charged mediator (*e.g.*, osmium bipyridine, hexacyanoferrate), the charge can be amplified on the gold gate through the action of the peroxidase, which changes the charge of the mediator.

The electronic charge detector structure of the present invention is then specifically designed to sense and report the presence of this positive or negative charge increase. The sample can be detected in an aqueous, dry or a sol-gel format on the transistor gate. Chitosan, polyacrylamide, or other gel forming materials can be used to produce a gel on the surface of the detector gate.

E. Signal Test

To test signal generation, test targets are subjected to the procedures described above. Samples are evaluated using different buffers, times and temperatures to determine parameters for optimal generation of the signal molecules. Signal molecule generation was verified using denaturing polyacrylamide gel electrophoresis or fluorescent labeling.

II. Working Embodiment of a Universal Signal Generation Process

FIG. 2 illustrates one embodiment of a process for detecting a biomolecule using RNA polymerase. Referring to FIG. 2, a reaction vessel **100** is provided for receiving and attaching a biological sample. The vessel can be a commercially available microplate or microtubes, or can be

fabricated using any convenient means to define a structure that is suitable for containing and fixing a predetermined amount of biological substance.

Biological target material **101** and non-target substance **102** can be attached to the reaction vessel **100** using a non-specific or specific binding capture probe as described above. In the case of FIG 2, the reaction container itself serves as the capture probe with a material that binds biological materials in a nonspecific way. Operatively coupling the biomolecules to the reaction vessel 100 can be accomplished using a commercially available protein or carbohydrate binder, a filtration binder or other nonspecific binders or biological materials. In other embodiments, the capture probe includes both a nonspecific binder to the reaction chamber and a target capture component.

The signal probe includes a DNA Template component **105** which allows *in vitro* transcription of DNA. Component **105** is linked through the 104 linkage to the recognition component **103**, which recognizes target substance **101**. The DNA template **105** sequence can be varied to produce the desired peptide or RNA aptamer and any biomolecule **103**, such as a nucleic acid, a protein, a peptide or other biomolecule, can serve as the recognition component.

Referring to FIG. 3, after the recognition components **103** have interacted with a corresponding target substance **101**, any unbound signal probes may be washed from the system leaving only the **103/101** adducts. Signal molecules are produced from the DNA template by *in vitro* transcription in the presence of enzyme-1 **106**, T7 RNA polymerase, enzyme-2 **107**, polyA polymerase, and a reaction solution **108** containing NTP's. The resulting signal molecule 109 consists of an RNA aptamer head and a polyA tail.

A molecular layer (such as a monolayer) coupled to a detection surface **110** and an electronic charge detector structure **111** (FIG. 4) includes spacer molecules **112** and affinity binding molecules 113. The spacer molecules **112** are substantially non-conductive molecules, and are used to prevent non-specific binding on the detection surface. The spacer molecules typically are small organic molecules or organic polymers such as polyethylene glycol as described above. Affinity binding molecules **113** recognize and capture the RNA aptamer head of signal molecules **109** through an affinity binding reaction. Affinity binding molecules **113** are chosen generally based on their affinity for a given RNA aptamer. Affinity binding molecules **113** can be formed of materials such as biomolecules and/or organic molecules.

In certain embodiments, spacer molecules **112** and affinity binding molecules **113** are covalently bonded to the detection surface **110.** In other embodiments, the spacer molecules **112** and affinity binding molecules **113** can be coupled to the detection surface **110** through other conventional bonding methods and can be formed either directly or indirectly.

Finally, as shown in FIG. 4, the reaction sample from reaction vessel **100** (which is shown in FIG. 3) is applied to a detection surface or surfaces **110**. The interaction between affinity binding molecules **113** and signal molecules **109**, with their attached polyA tails having negative charges, results in increased negative charge on the detection surface or surfaces **110**. The electronic charge detector structure **111** reports the presence of this charge increase.

Typically, the detector surface **110**, including its affinity binding molecules **113** and spacer molecules **112**, is surrounded by a containment area **120** (FIG. 5) into which the signal molecules produced in a reaction vessel **100** (FIG. 3) are introduced. As shown in FIG. 5, the reaction vessel **100** and containment area **120** can be coupled such that the signal molecules produced in reaction vessel **100** can be transported to containment area **120** (for example, by fluid flow induced by pressure, capillary action or vacuum). In a particular embodiment, the entire detection system including the reaction vessel and containment area can be incorporated into a microfabricated biochip using micofluidics technology. For example, with reference to FIG. 5, the reaction vessel **100** can be microfabricated as a small well on a biochip and interconnected with the containment area, which also can be a small well that houses the detector surface **110**. The wells in this embodiment are interconnected through a channel **123**, and flow through the channel can be regulated by a valve in this flow-through embodiment. An inlet channel **121** on the reaction vessel, and an outlet channel **125** on the detection chamber allow the movement of the sample to and from the biochip. One or more additional inlets and outlets **122** and **124** can be added to facilitate sample and reagent transfer within the system. Such microfabricated biochip embodiments can be highly multiplexed to permit higher throughput detection of biomolecules.

It is also possible for the reaction vessel and the containment area to be the same physical space, thereby further reducing the complexity of the system.

III. Electronic Charge Detector

As illustrated in FIG. 6, one embodiment of the electronic charge detector of the present invention comprises a detector surface **110** that includes a gold structure upon a silicon dioxide surface **114**. The gold structure can be implemented with a gold bump bond method or coated on the surface using a plating or sputtering process common in the industry. Detector surface **110** is electrically coupled to a metal conductor path **115** formed in surface **114**. Metal conductor path **115** can be formed of a combination of aluminum and other elements that are common in CMOS manufacturing processes. Metal conductor path **115** terminates on, and is electrically coupled to, a polysilicon gate **116** of a field effects transistor (FET) **117**. In some embodiments, the FET transistor **117** can be any of a number of traditional CMOS.

Amplifier electronics **118** are electrically coupled to transistor **117** and detect the change in charge at the detector surface **110** that arises from the specific binding of signal molecules **109** to affinity binding molecules **113**. Digital analysis circuitry **119** is electrically coupled with amplifier electronics **118** and is configured to perform accounting, ratio, summation, threshold and other operations appropriate for analysis. In other embodiments, circuitry **119** can be configured to drive a digital communication network. In particular embodiments, the digital analysis circuitry **119** is implemented into a microprocessor. In some embodiments, the elements comprising the electronic charge detector reside on a single semiconductor circuit.

Referring now to FIG. 7, after a charge is picked up by the detector surface D, a comparator circuit analyzes the charge. The analysis or operation of the comparator circuit comprises a reference

voltage that drives the gate of M4 (V_{ref}). The charge to be detected is placed on the gate of M2 (D). The placed charge ΔQ changes the voltage of D as $\Delta V = \Delta Q/C$. With the clock (CK) low, the outputs Q and QN are both high. As CK rises, N1 and N2 both begin to rise. A difference in voltage on the two inputs, D and V_{ref}, causes an imbalance in the voltage of N1 and N2. If D is higher than V_{ref}, as would be the case for positive ΔQ, M2 has greater channel conductance than M4. This in turn allows N2 to rise faster than N1. Since N2 rises higher than N1, M1 has less channel conductance than M3 and the rise of N2 is accelerated relative to N1 (positive feedback). Soon thereafter, M1 shuts off, allowing N1 to be pulled to ground and N2 to pull to the voltage on CK. QN then transitions low while Q remains high. If V_{ref} is higher than D, M4 has greater channel conductance than M2 and in a fashion opposite to that described above, and Q transitions low while QN remains high.

Transistor M1, M2, M3, and M4 sizing can be determined for any particular CMOS process and proper circuit operation and sensitivity can be verified through circuit simulations such as HSPICE (Synopsys, Inc., Mountain View, CA). Such sizing and simulations have been performed for the AMIS 0.5 micron process using AMIS' transistor models.

The voltage on V_{ref} and D is advantageously above the threshold voltage of M2 and M4. In order to precisely control the placed charge threshold an initial controlled bias charge is coupled onto the gate of M2 before the detection sequence commences. The ability to control the initial bias charge and therefore voltage of the gate also allows the DC bias of the dynamic voltage range to be sufficiently high to enable the use of a normal (relatively high) threshold MOS transistor processes. The same technique also will work with low threshold transistors, which may be utilized in order to gain other process or performance advantages. As the gate of M2 is floating, charge can be coupled onto the gate in a manner related, but not identical to, erasure of an EPROM. During this initial D gate biasing the M2 transistor substrate is held at some fixed bias voltage while the transistor region is excited by ultraviolet radiation. This excitation produces charge tunneling through the gate oxide between the substrate and gate such that the gate and substrate are at the same electrical potential.

Shown in FIG. 8 is a complementary circuit to that shown in FIG. 7, whereby negative captured charge ΔQ will be detected on the detector surface D. With the clock (CKN) high the outputs Q and QN are both low. As CKN falls, both N3 and N4 begin to fall. A negative ΔQ will result in a lower voltage on D than V_{ref}, M6 is has greater channel conductance than M8, and N4 falls faster than N3. M5 is has less channel conductance than M7 and the fall of N4 is accelerated relative to N3 (positive feedback). Soon thereafter, M5 shuts off, thereby allowing N3 to be pulled to Vdd and N4 is pulled to the voltage on CKN. QN remains low while Q transitions high. If V_{ref} is lower than D, M8 is has greater channel conductance than M6 and in a fashion opposite to that described above, and Q remains low while QN transitions high.

Transistor M5, M6, M7, and M8 sizing can be determined for any particular CMOS process and proper circuit operation and sensitivity can be verified through circuit simulations such as HSPICE (Synopsys, Inc., Mountain View, CA). Such sizing and simulations have been made for the AMIS 0.5 micron process using AMIS' transistor models.

In other embodiments, a plurality of detection surfaces **110** (FIGS. 4 & 5) and (FIG. 6 or FIG. 7) arranged in an array provide the surfaces for receiving a signal molecule. For example, in one embodiment, illustrated in FIG. 9, a layout for a **128** pad system is implemented on a 2,500 x 2,000 micron die. One half of the device has detector surfaces **110** tied to the gates of P-MOS devices as shown in FIG. 8 and the other half have detector surfaces **110** tied to the gates of N-MOS devices as shown in FIG. 7. The number of devices on a single chip is limited only by the foundry process, the size of the gold detector surfaces, the separation of the gold detector surfaces and packaging considerations. The device shown in FIG. 9 was implemented in a 0.5 micron CMOS process fabricated at AMI Semiconductor (Pocatello, ID). The device could have been easily implemented in 0.35 micron or smaller feature electronics.

Circuit topologies other than those discussed above apply equally well to practicing the disclosed method and device. An important consideration is to have a comparator with a relatively high impedance, such as at least about 100 giga-ohms, and low capacitance input, such as about 25 femto-farads. A single MOS transistor gate input topology satisfies these criteria.

Various circuits capable of functioning as a comparator for use with the presently disclosed embodiments are described in a number of printed publications, some of which are listed in the Appendix. The documents listed in the Appendix are all specifically incorporated by reference herein.

The following examples are intended to illustrate certain features of the present invention. A person of ordinary skill in the art will appreciate that the disclosed embodiments of the present invention are not limited to the features exemplified by these working examples.

IV. Examples

Example 1 - DNA Detection

A. Preparation of a Signal Probe for DNA Detection

For the purposes of DNA detection, both T7-A30 and T7-P22B signal probes were modified to include a recognition component to allow the specific recognition of a particular DNA sequence. In one embodiment, a commercially available cloning vector (pbluescript SK +, described below) was used as the target. The recognition component was designed to hybridize to the 1576 base region of the target, which was prepared as described below, and was commercially synthesized to have the sequence of 30 bases shown immediately below:

(SEQ ID NO: 27)
5'- /Phos/CAG GAT TAG CAG AGC GAG GAA AAA ACT TAA -3'

The above oligomer was ligated to a DNA template component by treating the template with EcoRI and then ligating the two oligomers using T4 DNA ligase. This produced a DNA template with a single-strand tail on the 5' end.

B. Preparation of Test DNA Target Molecule

A test DNA target was prepared from a commercially available pbluescript SK + plasmid having 3,000 base pairs. The plasmid was linearized using the Hind III restriction enzyme and then denatured to a single strand using sodium hydroxide.

C. Preparation of a Capture Probe for DNA Detection

A capture probe was designed to interact with a specific region of pbluescript SK in a region upstream from the SK tail 1567 at base 1096. The SK-1096 thus served as the target capture component (TC). This biomolecule was commercially synthesized with biotin attached to the 5' end to allow attachment of the target to a solid phase. In this instance, the biotin serves as the Attachment Component (AC) of the capture probe and specifically binds with streptavidin magnetic beads to attach the probe to the solid phase. The following sequence was used for the capture probe:

(SEQ ID NO: 28)
5'- /Bio/CTC ACT CAA AGG CGG TAA TAC GGT -3'

D. DNA Hybridization Signal Test

To test the hybridization and signal generation, the test target was mixed with the signal probes and the capture probe and incubated at 65° C for 20-30 minutes and 3 hours at 42°C or 37°C. At the end of the incubation, the mixture was mixed with streptavidin magnetic beads and the streptavidin-biotin-DNA complex was purified using a magnet separator. A sample of the purified material was then subjected to PCR and run by gel electrophoresis to verify hybridization with the signal probe. A second sample of the material (without prior PCR) was used for signal generation and amplification using T-7 RNA polymerase and Poly A polymerase. Incubating the samples in SSC (high salt concentration buffer) for 3 hours proved to be advantageous for generating signal molecules. Generation of the RNA aptamer-poly A signal molecule via the T7-A30 signal probe and the P22 B RNA aptamer via the T7-P22B signal probe were verified using denaturing polyacrylamide gel electrophoresis.

Using AmpliScribe™ T7-Flash™ Transcription kit (EPICENTRE, Madison, WI) increased production of the RNA aptamer in a shortened period of time. For example, prior to using the transcription kit, this process required about 3 hours, whereas using the kit reduced this process time to less than 30 minutes.

EXAMPLE 2 - Protein, Carbohydrate and Whole Cell Detection

Protein and/or carbohydrate detection can be achieved using solutions or solid state samples and is also the basis upon which whole cells are detected. Whole cells of eukaryotic or prokaryotic origin, as well as virus particles, can be targeted by these methods since they typically have unique molecules, such as proteins and glycoproteins, on the outer surfaces of their cell membranes/walls. Typically, the recognition component of the signal probe and the capture component of the capture probe are chosen to bind specifically with a 3-dimensional structure of a protein or carbohydrate, either in solution or on the surface of the targeted cell.

A. Preparation of Signal Probe for Protein/Carbohydrate Detection

Either the T7-P22B or the T7-A30 template is used as the signal component of the signal probe for proteins or carbohydrates. However, the template sequence used in this probe can be varied to allow generation of different signal molecules (RNA aptamer, tag peptide) representing each specifically targeted protein or carbohydrate

The signal probe is prepared by conjugation of the DNA template to a recognition component. For example, the DNA template can be modified to include an amine or a sulfhydryl at the 5' end, and linkage to the protein recognition probe can be accomplished using direct chemical conjugation.

Alternatively, the signal component is linked to the recognition component using commercial linkers that allow the direct covalent linking of the DNA of the signal component to the protein and/or RNA of the recognition component. The recognition component can be, for example, a protein, a peptide, or a DNA/RNA aptamers that specifically binds with a target.

In a second embodiment, a protein and/or carbohydrate sample including the desired target is first biotinylated using a commercially available biotin labeling kit (N-hydroxysuccinimide-Polyethylene Oxide-Biotin, NHS-PEO4-Biotin, Pierce Biotechnology, Rockford, Ill.). In a particular embodiment, the biotin labeling kit is used to label the outer membrane proteins of E. coli. This is achieved by harvesting the bacteria from an overnight culture, re-suspending in PBS buffer at a selected density and then incubating with 2 mg of NHS-PEO4-Biotin for 30 minutes. Unreacted biotin is removed via centrifugation and washing. Subsequently the biotin labeled proteins and carbohydrates can be directly captured on the detector surface by the use of specific antibodies as the affinity binding materials. The biotinylation serves to both develop the signal molecule and to capture the target material.

B. Preparation of Capture Probe for Protein and Carbohydrate Detection

As described earlier, a capture probe can be used to capture a target and bind it to a reaction vessel, and thus facilitate the interaction of the signal probe with the target. As with DNA, in some cases the target molecule will be immobilized in a reaction vessel, such as a test tube, a column or a plate well. As a result, the capture probe serves to bind the target to a surface in the vessel, such as

by being attached to at least a portion of, and perhaps substantially completely coating, vessel component surfaces, such as glass slides, columns, or magnetic beads. A Streptavidin:Biotin interaction useful for the DNA capture probe also can be used.

As with the recognition component of the signal probe, the capture probe is designed to attach to the target at a specific 3-dimensional site, and in most cases at a different interactive site than that of the signal probe. As with the recognition component of the signal probe, the biomolecule used for the capture component of the capture probe will, in most cases, be proteins, peptides or DNA/RNA aptamers. In some cases, the primary role of the capture probe is to immobilize the target to allow interaction with the signal probe without the need for specific recognition. In these situations the interaction can be nonspecific binding, as can be achieved through test wells, membranes and other materials. It also is possible that the sample could be a biopsy or a slice of a mammalian or plant cell affixed to a glass slide using traditional techniques.

C. Procedures

Particular examples, for detecting proteins, carbohydrates and whole cells are given below.

1. Protein (*Staphylococcus aureus* Enterotoxin B and *Yersinia enterocolitica* Invasin)

In an embodiment of a signal probe for *Staphylococcus aureus* Enterotoxin B the recognition component can consist of two parts, either protein A or G, which react universally with antibodies, and an antibody specific for the target, namely, Enterotoxin B. Protein A or G can be obtained commercially (Sigma-Aldrich, St. Louis, MO) and are covalently linked to a DNA template component using a commercially available linker or through a SHA:PBA linkage as described above. After capture of the Entertoxin B using a 96 well antibody plate as described below, the captured target is reacted with an antibody that specifically binds enterotoxin. In this case, Monoclonal Toxin B Antibody specific for the enterotoxin (US Biological, Swamscott, MA) is used. Subsequently, the linked signal probe reacts with the specific antibody through the protein:protein interaction between protein A or G and the antibody. Alternatively, the Protein A or G coupling may be eliminated if the antibody is directly linked to the signal component. The antibody component of the signal probe then interacts with a specific site on the target.

In another embodiment for selectively detecting invasin (a cell surface protein common in organisms such as *Yersinia enterocolitica*) an RNA aptamer or peptide that specifically binds invasin serves as the recognition component of the signal probe and is directly linked to the signal template component, which in this embodiment is also a DNA template. Invasin was cloned and used to develop specific RNA aptamers or peptides using SELEX and Ribosomal Display.

For detecting Enterotoxin B or invasin, the targets are captured using a 96-well antibody test plate in lieu of using a capture probe to attach it to a surface of the reaction vessel. Enterotoxin B - or invasin is captured on the plate and then treated with either the Monoclonal Toxin B Antibody specific for the enterotoxin (US Biological, Swamscott, MA) or the RNA aptamer or peptide specific for invasin. Subsequently, the plate is treated with the invasin- or enterotoxin B-specific signal probe as described above and the plate is then washed to remove any unbound signal probe. Subsequently,

the plate is then treated with RNA polymerase and Poly A polymerase to generate the signal molecules for detection by the detector as described above.

2. Carbohydrate (O-Antigen Polysaccharide)

The O-antigen polysaccharide, which is a cell wall component of *E. coli* 0157:H7, is the target. *E. coli* 0157:H7 lipopolysaccharide (LPS) which contains the O-antigen is extracted from whole cells using phenol. The LPS can be captured using polystyrene plates that non-specifically bind hydrophobic materials such as LPS. The trapped LPS is then treated with a commercially available antibody to O157-antigen (Maine Biotechnology Services, Inc., Portland, ME). Subsequently the antibody/LPS is treated with the protein A or G signal probe described above. As before, once the unbound signal probe is washed from the sample, RNA polymerase and Poly A polymerase are added to generate the signal molecules. Alternatively, the O-antigen can be subjected to SELEX procedures to develop specific RNA/DNA aptamers that can be used on the capture probe and/or the signal probe for recognition.

3. Whole cells (*E coli* 0157:H7 and *Yersinia enterocolitica*)

In this embodiment, whole microbial cells are detected using signal probes that include a recognition component that binds specifically with known cell surface molecules. In the case of *E coli* 0157:H7, for example, the target can be the O-antigen as described above. With *Yersinia*, the target is invasin, a cell surface protein. *E. coli* 0157:H7 is detected using a signal probe that recognizes the O-antigen target. The cells are immobilized through filtration or columns, perhaps enhanced using a capture probe of the O-antigen antibody or the developed aptamer. The captured cells are then subjected to the signal probe with the attached antibody to the O-antigen, washed and the attached signal probe used to generate the signal.

For *Yersinia*, invasin has been cloned and used to develop specific RNA aptamers or peptides using SELEX and Ribosomal Display. The resulting aptamers or peptides can be used to develop capture and signal probes as described above.

Aptamers generated as described may be used to develop a whole cell sensor where the cells are captured directly on the semiconductor gate using aptamers or antibodies placed directly on the gate surface instead of peptides. The whole cells could provide adequate charge for the detection without the need for amplification of the charges as is described for the other procedures.

D. Signal Test

To test signal generation, test targets are subjected to the procedures described above. Samples are evaluated using different buffers, times and temperatures to determine parameters for optimal generation of the signal molecules. Signal molecule generation is verified using denaturing polyacrylamide gel electrophoresis. In addition, a transcription kit, such as the AmpliScribe™ T7-Flash™ Transcription kit (EPICENTRE, Madison, WI) can be used to increase production of the RNA aptamer in a shortened period of time. Signal molecules are then detected using the devices described above. Although times in excess of 30 minutes are often needed to develop an adequate number of signal molecules for charge detection using current techniques (see, for example,

Sambrook, J. and D.W. Russell, "Synthesis of single-stranded RNA probes by in vitro transcription", Molecular Cloning, 3rd edition, Vol. 2, Protocol 6, 2001, pp. 9.29-9.37), less than 10 minutes are needed to generate enough signal molecules for detection by the semiconductor device described above.

Having illustrated and described the principles of the invention in exemplary embodiments, it should be apparent to those skilled in the art that the illustrative embodiments can be modified in arrangement and detail without departing from such principles. In view of the many possible embodiments to which the principles of the invention can be applied, it should be understood that the illustrative embodiments are intended to teach theses principles and are not intended to be a limitation on the scope of the invention. We therefore claim as our invention all that comes within the scope and spirit of the following claims and their equivalents.

APPENDIX:

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